PRODUCTION RESEARCH PAPERS

# Purification and Properties of an Acid Phosphoprotein Phosphatase from Lactating Bovine Mammary Gland with Activity Toward Phosphotyrosine

## ABSTRACT

An acid phosphatase has been partially purified from lactating bovine mammary gland. Properties of this enzyme were compared with those of a well-characterized phosphoprotein phosphatase from bovine spleen. The two enzymes were similar in their activation by sulfhydryl reagents and inhibition by metal chelating agents. Both enzymes rapidly hydrolyze ATP and aromatic phosphates and are relatively inactive toward alkyl phosphates; both are tartrate-resistant phosphatases. The mammary enzyme has a low Michaelis constant for $\alpha_{S1}$-casein (42 $\mu M$), and thus, like the spleen enzyme, appears to be a phosphoprotein phosphatase. Finally, the spleen and mammary enzymes displayed reactivity toward phosphotyrosine, a model substrate for phosphotyrosyl protein phosphatase. Thus, the phosphatases from spleen and mammary gland are quite similar in reactivity and could possibly be similar in function.

## INTRODUCTION

Acid phosphatases occur in a variety of mammalian tissues; these enzymes often seem to be nonspecific in nature and act on a variety of phosphomonoesters (1, 21). Many phosphatases, however, particularly phosphoprotein phosphatases, are more specific in nature; these enzymes participate in cellular regulation by reversing second messenger-regulated phos-phorylations of selected enzymes. Ingebritson and Cohen (12) reviewed this area in detail. Historically, acid phosphatase has been recognized as a component of milk (21). The milk enzyme has been partially purified and characterized by several groups (2, 5, 13). In reviewing the properties of this enzyme, Bingham et al. (5) and Shahani et al. (21) concluded that it should be classified as a phosphoprotein phosphatase. Similar phosphoprotein phosphatases from other tissues have been thought of as not being involved in cellular regulation; however, Lau et al. (14) recently presented evidence that such an enzyme from bone may be a regulatory phosphotyrosyl phosphatase. These latter enzymes participate in cellular development by reversing the action of tyrosine kinases (15, 16, 17). The objective of this work was to purify the mammary acid phosphoprotein phosphatase and to clarify the nature of the enzyme.

## MATERIALS AND METHODS

$\alpha_{S1}$-Casein was prepared as previously described (3, 22). p-Nitrophenyl phosphate (p-NPP) (dicyclohexylammonium salt), O-phospho-L-tyrosine and other biochemicals were purchased from Sigma Chemical Company (St. Louis, MO).[3] Mammary glands from cows were obtained from J. E. Keys and A. J. Guidry (USDA, Beltsville, MD).

### Analytical Methods

Phosphorus determinations of the protein were carried out as previously described (3, 4). Total protein was determined by the method of Lowry et al. (18) with bovine serum albumin as the standard.

### Preparation of Enzymes

The preparation of mammary gland phosphoprotein phosphatase was similar to the method of Revel and Racker (20) for preparation of spleen phosphoprotein phosphatase. Mammary glands from freshly slaughtered

lactating cows were obtained and immediately put on ice. The period between the death of the cow and the beginning of the procedure was never more than 2 h. All experiments were conducted at $5°C$ unless noted. The procedure is outlined in Figure 1; assays of pertinent fractions are in Table 1. Three 400-g portions of mammary gland were diced, put through a meat grinder, and then homogenized in a Waring blender in 1 L of .5 $M$ NaCl-.2 $M$ acetate buffer pH 5.0 for 3 to 5 min. The homogenate was squeezed through one layer of coarse and then one layer of fine cheesecloth. Centrifugation was at 1000 × $g$ for 15 min at $5°C$.

The homogenate was brought to 20% saturation with $(NH_4)_2SO_4$, stirred for 15 min, and then centrifuged at 16,000 × $g$ for 20 min; the precipitate obtained contained little activity and was discarded. The supernatant was then brought to 35, 70, and 90% saturation, repeating the process three times. Although the 70% precipitate had the highest specific activity, the bulk of enzyme activity was in the 20 to 35% precipitate. Both ammonium sulfate precipitates were resuspended in a small volume of water and dialyzed until free of sulfate ion. In each case, precipitates formed upon dialysis. The dialysates were centrifuged at 16,000 × $g$ for 20 min. The supernatant from the dialyzed 20 to 35% precipitate had little activity and was discarded. The supernatant from the 35 to 70% step contained a significant amount of activity (Step 3, Soluble II-A, Table 1) and was retained. The insoluble pellets were extracted with homogenization buffer; these extracts (Step 3, Precipitates I-A and II-A) had good activity and were also retained. All three fractions were subjected to heat and protamine sulfate treatment in accordance with the method of Revel and Racker (20). The soluble fractions from these steps, which had enzymatic activity, were pooled and brought to 80% saturation with ammonium sulfate and centrifuged at 8000 × $g$ for 15 min. The precipitate, containing the enzyme, was resuspended in homogenization buffer. At this point purification was 33- to 37-fold (data not shown).

The enzyme solution was dialyzed against .2 $M$ acetate buffer, pH 5.0, until free of sulfate ion; the dialysate was adjusted to pH 6.7 with NaOH and clarified by centrifugation at $5°C$ for 30 min at 100,000 × $g$. The supernatant was then stirred with Amberlite IRC-50 (1 g/100 ml), a weak cation exchange resin, equilibrated as previously described (5) at pH 6.0. The resin was washed with several volumes of water and the enzyme was eluted with 1 $M$ NaCl. The eluate was then passed through a column of Sephadex G-50 equilibrated in homogenization buffer and the active fractions retained.

## Phosphatase Assays

Kinetic studies of inhibitors and activators using p-NPP as a substrate were carried out in the following manner: .5 ml of a 30 m$M$ p-NPP solution was added to 1.5 ml of 10 m$M$ pH 5.8 sodium acetate buffer; 1.0 ml of inhibitor, activator, or buffer; the reaction was started by addition of 25 $\mu$l of enzyme. The time course of the mixture was assayed at 410 nm with a Gilford Model 2000 spectrophotometer; the molar extinction coefficient of p-nitrophenol at this pH is 1540. Assays for o-carboxyphenyl phosphate were conducted in a similar fashion except a wavelength of 300 nm and a molar extinction coefficient of 3400 were used, and a nonenzymatic blank subtracted (23). Spectrophotometric assays for phosphotyrosine hydrolysis were by the method of Farrell and Dower (10). Final concentrations of inhibitors and activators ranged from .3 to 20 m$M$. Most assays were also run with phosphoprotein phosphatase from spleen as the hydrolyzing enzyme to compare its properties to the mammary enzyme. Studies of the enzyme at various pH were conducted in a different manner; .5 ml of buffer (.2 $M$ acetate titrated to the appropriate pH with NaOH), .5 ml of 10 m$M$ p-NPP in water, and 25 $\mu$l of enzyme were incubated at room temperature for 7 min. Then 2.0 ml of .1 N NaOH was added to stop the reaction and develop the color. Results are reported as change in absorbance at 410 nm per minute.

For the study of substrates by release of inorganic phosphate, the following procedure was used. Substrate, 1.0 ml, was incubated in a glass centrifuge tube in .10 m$M$ acetate buffer adjusted to pH 6.0 for 30 min at $37°C$ with and without 25 $\mu$l of enzyme. Then enzyme was added to the blank and both reactions stopped by addition of .5 ml of .02 $M$ silicotungstic acid. The tubes were clarified at 2000 rpm in a clinical centrifuge and the supernatants taken for analysis of inorganic phosphate by the method of Martin and Doty (19).

TABLE 1. Properties of the various fractions obtained in the purification of mammary acid phosphatase.

| Step | Description | Volume | Protein | p-NPP Activity[1] | | PPPase Activity[2] | |
|---|---|---|---|---|---|---|---|
| | | (ml) | (mg/ml) | (total units × $10^{-3}$) | (units/mg protein) | (total units × $10^{-3}$) | (units/mg protein) |
| 1 | Homogenate | 2800 | 44.5 | 440 | 3.52 | 10.3 | 87.6 |
| 2 | 20 to 35% $(NH_4)_2$ $SO_4$ Precipitate | 1200 | 96.6 | 281 | 2.42 | 10.0 | 86.6 |
| 2 | 35 to 70% $(NH_4)_2$ $SO_4$ | 500 | 16.6 | 66.0 | 7.95 | 6.20 | 147 |
| 3 Precipitate I-A | Extract of 20 to 35% ppt[3] after dialysis | 500 | 8.50 | 54.5 | 12.8 | 1.28 | 300 |
| 3 Precipitate II-A | Extract of 35 to 70% ppt after dialysis | 250 | 5.30 | 30.5 | 23.0 | .61 | 456 |
| 3 Soluble II-A | Soluble fraction 35 to 70% ppt after dialysis | 900 | 6.75 | 112 | 18.4 | 2.11 | 346 |
| 4 | Pooled enzyme after heat and protamine sulfate | 325 | 2.50 | 107 | 132 | 2.28 | 2800 |
| 5 | IRC-50 eluate[4] | 13 | 2.05 | 8.65 | 325 | .14 | 5370 |

[1] For p-NPP assay a unit is defined as a nanomole of p-nitrophenol liberated per minute at 25°C; no reducing agents are present.

[2] For phosphoprotein phosphatase (PPPase) activity a unit is defined as a pico mole inorganic phosphate liberated per minute at 37°C; the specific activity is expressed in milliunits per milligram for clarity. Casein concentration was 15 mg/ml; no reducing agents were present in the assay.

[3] ppt = Precipitate.

[4] The pooled supernatant from Step 4 was divided into three equal volumes and each processed separately. Results here are average of two trials; multiply by 3 for total yields.

## Purification of Phosphatase from Mammary Gland

The classical preparation of phosphoprotein phosphatase of Revel and Racker (20) was applied to mammary gland. Their method had to be partially modified; the new concentrations of salt saturation 35 and 70% gave better separation of the enzyme, and their DEAE-cellulose step resulted in losses of enzyme activity for mammary preparations. In another preparation, the crude enzyme was adsorbed onto IRC-50, eluted with 1 $M$ NaCl, and passed through Sephadex G-50. Although the specific activity increased with these steps, yields were poor. The final purification was 90-fold with a total yield of 3%. The results of these steps are summarized in Table 1. Initially the activity of the enzyme was followed using $p$-NPP; however, the reactivity toward casein was also studied at each step to determine if $p$NPP-ase and phosphoprotein phosphatase (PPPase) activites were copurifying. Table 1 shows that this was the case. The reactivity of the mammary gland enzyme was then compared with a preparation of phosphoprotein phosphatase from bovine spleen made directly by the procedure of Revel and Racker (20).

## Comparison of Activators, Inhibitors, and pH Optima

The mammary acid phosphatase was compared with the spleen enzyme to determine the effects of known activators and inhibitors of the latter enzyme (11, 20). The spleen enzyme is a metal-containing enzyme; the chelating agents EDTA and bipyridal do not directly inhibit the enzyme, but preincubation for 10 min with these agents results in a loss of activity for the spleen and mammary enzymes (Table 2). Addition of reducing agents enhances the activity of the spleen enzyme and the mammary enzyme is comparably stimulated (Table 2). The two preparations, as shown in Table 2, behaved similarly toward inhibitors, which are chelators, and toward activators, which are reducing agents. Because of the activation achieved by 2-mecaptoethanol, this reagent was included in most subsequent assays. Neither enzyme is inhibited by DL-tartrate, which places them both in the class of tartrate-resistant acid phosphatases. The most potent inhibitor of both enzymes is vanadate, with a median inhibition ($I_{50}$) of .13 m$M$.

The pH activity curves of the mammary gland acid phosphatase were compared with those of the spleen enzyme using $p$-NPP and $\alpha_{s1}$-casein as substrates. As shown in Figure 2,

TABLE 2. Effects of activators and inhibitors on the reactivity of spleen and mammary acid phosphatase toward $p$-nitrophenyl phosphate ($p$-NPP).[1]

| Additive | m$M$ Concentration | Mammary | Spleen |
|---|---|---|---|
| None | | 100 | 100 |
| Thioglycollate | 2 | 100 | 100 |
| | 10 | 171 | 135 |
| 2-Mercaptoethanol | 2 | 161 | 161 |
| | 10 | 171 | 171 |
| Ascorbate | 2 | 100 | 100 |
| | 10 | 131 | 150 |
| Dithiothreitol | 2 | 126 | 121 |
| | 10 | 126 | 143 |
| EDTA preincubation | 10 | 57 | 64 |
| Not preincubated | 10 | 100 | 100 |
| Bypyridal preincubation | 1 | 82 | 80 |
| Not preincubated | 1 | 100 | 100 |
| Tartrate preincubated | 2—20 | 100 | 100 |
| Not preincubated | 2—20 | 100 | 100 |
| Vanadate preincubated | .13 | 48 | 56 |
| Not preincubated | .13 | 46 | 52 |

[1] Relative activity using the standard $p$-NPP assay at pH 5.8.

TABLE 3. Comparison of the substrate specificities of bovine mammary and spleen phosphatases.

| Substrate | Enzyme activity[1] | |
| --- | --- | --- |
| | Mammary | Spleen |
| | (nmol $P_i$ released/ 30 min) | |
| ATP | 165.0 | 188.0 |
| Flavin mononucleotide | 67.7 | 59.0 |
| o-Carboxyphenyl phosphate | 53.2 | 49.9 |
| $NADP^+$ | 37.3 | 56.9 |
| Glucose-6 phosphate | 19.6 | 24.7 |
| β-Glycerol phosphate | 12.7 | 9.4 |
| Pyridoxal phosphate | 8.9 | 11.4 |
| Phosphoserine | 8.8 | 22.2 |

[1] Subtrates were assayed for release of inorganic phosphate by incubation for 35 min at 37°C with subtraction of a zero time blank described in Materials and Methods; 2-mercaptoethanol included at 2 mM. $P_i$ = Inorganic phosphate.

the pH activity curves of these two enzymes are rather broad and quite comparable for both substrates. When added directly to the reaction mixture, the following metal ions were without effect: $Na^+$, $K^+$ at 10 mM; $Mg^{2+}$, $Ca^{2+}$, $Ba^{2+}$, $Sr^{2+}$, and $Mn^{2+}$ at 2 mM. A 2 mM $Zn^{2+}$ and $Cu^{2+}$ inhibited both enzymes. These data indicate a further similarity in reactivity for the two preparations.

### Comparison of Substrate Specificities

Table 3 shows a comparison of the relative reactivities of the spleen and mammary enzymes toward a variety of low molecular weight phosphate containing compounds. The reactivity of the spleen enzyme is in agreement with previous reports (11, 20); the spleen and mammary enzymes appear to be quite similar in their ability to release inorganic phosphate from these compounds.

Table 4 gives the reactivities as measured spectrophotometrically for hydrolysis of the aromatic substrates p-NPP, o-carboxyphenyl phosphate, and phosphotyrosine at pH 5.8, 5.5, and 6.0, respectively. All three substrates are reactive in the same relative order with both enzymes; p-NPP is most reactive and phosphotyrosine is least reactive. However, the spleen enzyme is six times more reactive than the mammary enzyme.

### Comparison of Michaelis Constants

The Michaelis constants ($K_m$) were obtained for both spleen and mammary acid phosphatases using p-NPP and $α_{s1}$-casein as substrates. The values given in Table 5 are comparable for both enzymes, and those obtained for the spleen enzyme are in good agreement with literature values (11, 20).

### DISCUSSION

The procedure of Revel and Racker (20) was used to purify the bovine spleen enzyme, and a preparation with specific activity comparable to their published values was obtained. This procedure was then applied to mammary tissue to determine if a similar enzyme were present. The procedure had to be modified as outlined in Figure 1. A minor modification was the percent ammonium sulfate needed as noted. However, in the case of the spleen enzyme,

TABLE 4. Relative reactivities of mammary and spleen phosphatases toward p-nitrophenyl phosphate, o-carboxyphenyl phosphate, and phosphotyrosine as measured spectrophotometrically.

| Substrate and pH optimum[1] | Enzyme activity | | | |
| --- | --- | --- | --- | --- |
| | Mammary | | Spleen | |
| | (nmol hydrolyzed/min per mg protein) | | | |
| | $\overline{X}$ | SE | $\overline{X}$ | SE |
| p-NPP pH 5.8 | 402 | 4 | 2450 | 40 |
| o-Carboxyphenyl phosphate pH 5.5 | 150 | 5 | 900 | 60 |
| Phosphotyrosine pH 6.0 | 68.5 | 5.5 | 432 | 13 |

[1] Assay conditions as described in Materials and Methods except substrate concentration was 3 mM; no reducing agents were present in the assay.

TABLE 5. Comparison of Michaelis constants of purified bovine mammary and spleen acid phosphatases.

| Substrate | Michaelis constant | |
| | Mammary | Spleen |
| --- | --- | --- |
| | $(\mu M)$ | |
| p-NPP[1] | 883 | 1380 |
| $\alpha_{s1}$-casein[2] | 42 | 65 |

[1] p - Nitrophenyl phosphate.

[2] Michaelis constant is based upon the monomer molecular weight of this protein; 2-mercapteothanol at 2 m$M$ included in the reaction.

when the ammonium sulfate precipitates are dialyzed, one fraction contains the bulk of the activity. Dialysis of the mammary preparations yielded three fractions with comparable activity (Table 1), indicating a possible inhibitor had been present before dialysis (perhaps residual inorganic phosphate); for example, the sum of activities from Precipitate II-A and Soluble II-A is greater than the total activity in the 35 to 75% ammonium sulfate cut from which they were derived. This was also true in the preparation of milk acid phosphatase (5). The three active fractions were treated with heat and protamine sulfate, and the soluble extracts were pooled (Figure 1). The DEAE-cellulose step, which was successfully used for the spleen enzyme, gave poor results for the mammary preparation with nearly total loss of activity. The cation-exchanger IRC-50, previously employed in the purification of acid phosphatase from milk (5), yielded an active and stable enzyme while removing colored impurities. After these steps the specific activity of the

Outline of Preparation of Mammary Acid Phosphatase

STEP 1   Homogenization and Centrifugation 1000 x g 15 min.

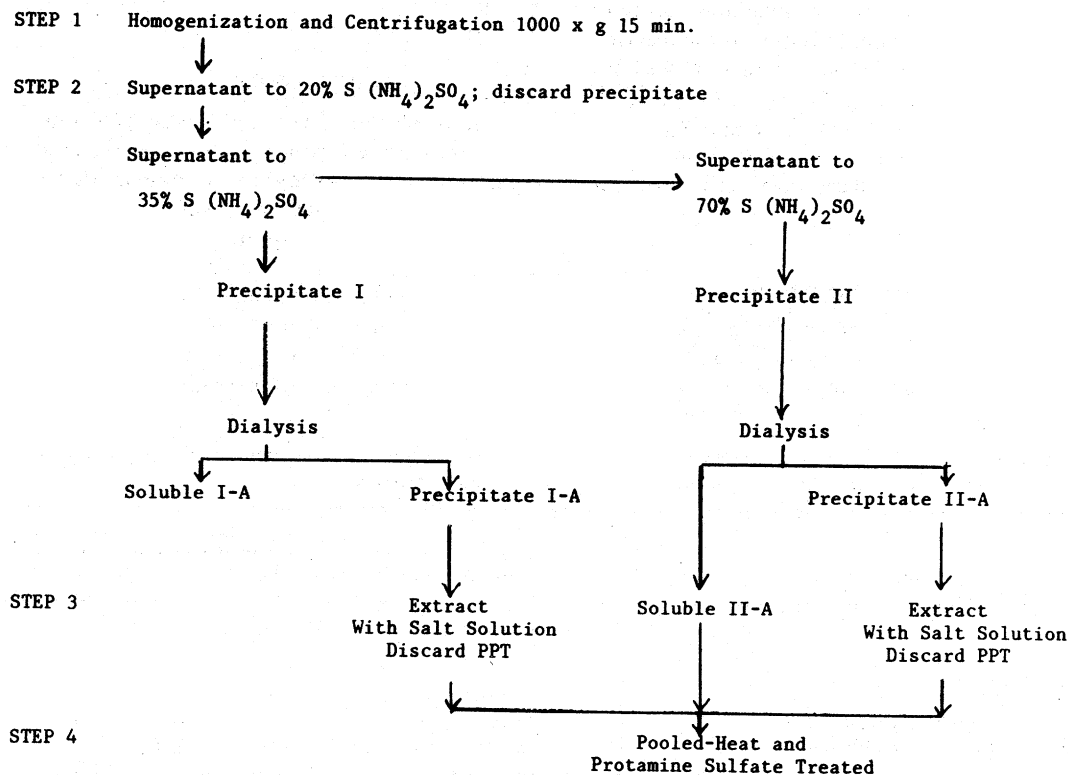STEP 2   Supernatant to 20% S $(NH_4)_2SO_4$; discard precipitate



Figure 1. Scheme for isolation of mammary acid phosphatase; percent S indicates the percent of saturation of the ammonium sulfate. PPT = Precipitate.

mammary enzyme was increased 100 times over homogenate. The specific activity of the purified spleen enzyme was overall about 6 times greater than that of the purified mammary preparation (Table 4).

Both mammary and spleen enzymes are reactive toward p-NPP and $\alpha_{s1}$-casein with pH optima in the range of 5 to 6, which classes them as acid phosphatases (Figure 2). The enzymes are similar in reactivity toward phosphate-containing sustrates as measured by the release of inorganic phosphate (Table 3). The higher affinity of the enzymes for the phosphoprotein $\alpha_{s1}$-casein, as indicated by their $K_m$ (Table 5), indicates that both enzymes are more properly classified as phosphoprotein phosphatases.

Phosphatases can also be classified as tartrate resistant or tartrate sensitive (1, 16); the later class of enzymes are thought to be lysosomal in nature and to be the general acid phosphatases of all cells (1, 16). In contrast, tartrate-resistant phosphatases have more specific metabolic roles. Both the spleen and mammary enzymes are tartrate-resistant, which indicates a more specific metabolic activity for these enzymes, although their exact functions are yet to be determined.

Both preparations respond positively to reducing agents and are inhibited by incubation with chelators. These two peculiar properties
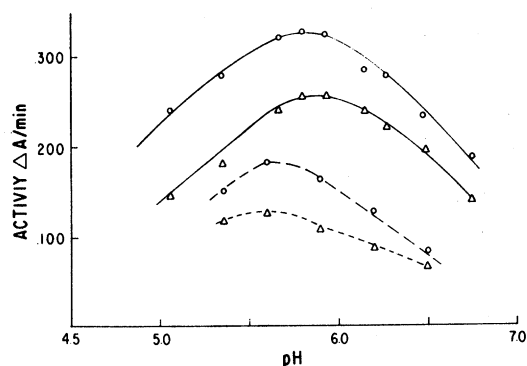


Figure 2. The pH activity (change in absorbance per minute) curves for the hydrolysis of p-nitrophenyl phosphate (p-NPP) and $\alpha_{s1}$-casein by bovine spleen and mammary enzymes; (-o-o-) spleen and p-NPP; (-Δ-Δ-) mammary and p-NPP; (- - -o- - -o- - -) spleen and $\alpha_{s1}$-casein; (- - -Δ- - -Δ- - -) mammary and $\alpha_{s1}$-casein.

have suggested to others (5, 8, 11) that metal ion oxidation and reduction could be associated with hydrolysis. The mechanism involved in this reaction, although under study for several years for the bone and spleen enzymes, is as yet unknown (6, 8, 14). Both spleen and mammary enzymes are not stimulated by added metal ions. Although $Zn^{2+}$ and $Cu^{2+}$ inhibit the reaction, this could be due to protein or substrate precipitation by these metal ions (9). Vanadate at .13 mM inhibits both enzymes; such inhibition is characteristic of phosphotyrosyl phosphatases (15).

The reactivity of both enzymes toward the model substrate phosphotyrosine and their inhibition by vanadate suggests phosphotyrosine-containing proteins or enzymes could play a role in the regulation of mammary growth and development, as has been postulated for other phosphatases displaying reactivity toward phosphotyrosyl proteins (6, 7, 15). Here only the reactivity toward the model compound was tested, but a similar phosphatase from rat spleen has been characterized as a specific phosphotyrosyl protein phosphatase (6), as has a similar enzyme from bovine bone matrix (14). The latter enzyme was reactive toward both phosphotyrosine and phosphotyrosyl residues in proteins. All of these enzymes are inhibited by vanadate.

In conclusion, comparison of pH activity curves, substrate specificities, $K_m$, and reactivities toward common inhibitors and activators indicates that the spleen and mammary phosphatases are highly similar in nature and that both enzymes should be classified as phosphoprotein phosphatases. The reactivity of both of these enzymes toward the amino acid phosphotyrosine opens the question of whether these proteins are specific for phosphotyrosine residues in selected proteins and thus could be considered regulatory enzymes.

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